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STANDARDIZATION OF PORTABLE CULTURE DEVICE FOR DETECTION OF MICROBES

INTRODUCTION:

Microorganisms are ubiquitous. Some of the organisms are pathogenic while some are non pathogenic. Pathogens originate and spread through various sources like food, water, air, clinical samples etc. Transmission of pathogenic microorganisms through these sources can lead to outbreak of diseases in a population. Food and water borne pathogens, for example, can get easily spread in a population with the advent of street food and ready to eat products. Early detection of pathogens in food and water samples can lead to prevention of spread of the disease. Hence detection of pathogenic microorganisms is significant in this context.

The Center for Disease Control and Prevention (CDC) estimated a global burden of food borne diseases (FBD) of about 3-5 billion which leads to 15 lakh deaths annually, which makes food safety a global concern. The FBD burden of India is estimated to be 1,000 lakh cases per year (Kristkova, Grace, & Kuiper, 2017). Water borne diseases cause of between 4-5 lakh deaths in children aged under five years. The probable sited reason for such incidences is failure to improve personal and home hygiene. Under-developed and developing countries have more incidences of food and water borne infections as compared to developed countries. Due to lack of well-equipped centralised laboratory facilities, many cases go unreported which leads to underestimation of the bioburden of such diseases (Olumide Odeyemi, 2016). Among the cases reported, most of the infections are known to be caused by *Staphylococcus aureus, Vibrio sp, Salmonella sp, Escherichia coli, Yersinia enteroclitica* and Norwalk-like virus, *Salmonella, Listeria monocytogenes, Shigella sonnei* and *Bacillus cereus.*

Thus, pathogen detection is important for health and safety reasons. Microbiological testing has two objectives viz. to confirm the absence of pathogens in food and water samples thus ensuring food and water safety and to enumerate total microbial load to monitor and verify product quality and shelf life. Thus, microbiological testing can be applied at all levels from production to consumption. Identification of pathogens at the point of contamination is considered as Hazard Analysis and Critical Control Point (HACCP).

The traditional methods for detection of pathogens include culture-based methods which are time consuming, laboratory and skilled labour dependent. (Camilla et al 2016). Sample transportation can be a major problem if laboratory facility is situated away from the site. To overcome the drawbacks of culture-based methods, pathogens are detected by advanced methods which include nucleic acid based or immunological based methods. Molecular methods are expensive and involve complex sample preparation procedures along

with the requirement of expensive reagents, enzymes, fluorescent dyes, instrumentation, sophisticated laboratory and skilled personnel which add on to cost of the detection process (Camilla et al 2016).

Due to the limitations in existing detection methods, efforts are needed to be made to improve the methods of pathogen detection. There is a need to develop detection devices which are economical. These systems should be robust, simple to use, stable within wide variety of operating conditions, suitable for on-site detection and made up of disposable material. Development of paper based microfluidic detection devices can help to overcome the limitations of the existing methods of detection of organisms. These devices follow the **ASSURED** criteria, which means that they should be **A**ffordable, **S**ensitive, **S**pecific, **U**ser friendly, **R**apid and **R**obust, **E**quipment free and **D**elivered to the needy. The current work will deal with the development of paper-based Portable Culture Device (PCD) that can be used for detection of microbes. This may help to overcome many problems which are associated with centralized testing facilities and aid in simplified on-site detection of microorganisms.

II. **RATIONALE**:

The current research work will be focused on standardizing a Portable Culture Device for the detection of microbes in various natural samples, especially food and water. Timely detection of microbes from such samples may, help in preventing outbreaks by, decreasing the bioburden of food and water. Portable Culture Device, due to its inherent nature of simple design and low volumes of reagents required, will be a cost-effective and user-friendly option to detect microorganisms. These properties of the device will suit the needs of developing countries which lack centralised testing facilities. Such devices will also find utility at houses, canteens, restaurants and local eateries. A colour grade card correlating the intensity of colour developed to the approximate microbial load in the sample can be ascertained. This type of analysis will allow detection and thus reporting of occurrence of microorganisms, if any, and will thus help policy makers to device control measures for the same.

III. LITERATURE REVIEW:

1. Importance of detection of pathogens:

The pathogenic microbes cause various infections. One of the infections is gastrointestinal tract infection which is caused by food and water. It has been reported that 1.5 billion people suffer from bacterial infections globally. Food and water are two very important sources of pathogenic bacteria. Foods (ready-to-eat products, dairy products, vegetables, fruits, nuts, confectionaries, seafood, meat and poultry) and water (drinking water) are found to be contaminated with pathogens. As a result of this, there is a need for development of rapid microbial detection devices with high sensitivity and specificity.

2.1 Bacterial water borne diseases:

These infections are one of the major public health concerns causing 22 lakh deaths annually. Consumption of contaminated water and poor sanitation are major sources of water borne pathogens. In India, the number of cases of gastroenteritis is 108 lakh/year out of which majority is caused due to unclean water and improper sanitation. The etiological agents for such infections are *Escherichia coli*, *Salmonella sps.* and *Shigella sps*. Potable water and water for recreational activities is generally monitored for the presence of fecal indicator bacteria. There are various reasons for presence bacteria in potable water like compromised water treatment, disinfection resistant biofilms in water pipelines. Water gets contaminated via the feco-oral route leading to contamination of water reservoirs, wells and groundwater.

2.2. Bacterial food borne diseases:

The food safety risks are high in India due to poor food safety controls, lack of monitoring and detection systems, lack of ability to handle food safety related issues and disasters (Article, 2018). In 2004, 9 lakh cases of acute diarrheal diseases with 2855 deaths were recorded (Sudershan, Kumar, Kashinath, Bhaskar, & Polasa, 2014). Integrated Disease Surveillance Programme (ISDP) of the Ministry of Health, Government of India estimates that 6921 of food borne infections were observed from 2008-17. Places like parties, hostel mess, army unit, marriages, etc where food is cooked in bulk are a major source of FBD. India spends almost Rs.1,78,100 crore which is around 0.5% of the country's gross domestic product (GDP) for management of FBD annually (Article, 2018).It is reported that most of the reported cases are caused by *Listeria monocytogenes, Salmonella typhi, Bacillus cereus, Vibrio cholerae and Escherichia coli*.(Safety, n.d., 2017)

3. Detection of organisms in food:

3.1.1.Microbial Culture dependent conventional methods:

Conventional culture based methods have two basic steps viz. isolating the organism on the selective medium and identification of the organism based on biochemical characterization (Mandal, Biswas, Choi, & Pal, 2011) .

3.1.2. Chromogenic media: These detection media utilize synthetic chromogenic enzyme substrates which aid in microbial detection. There are several commercially available chromogenic media. Following are a few examples:

3.1.2.1 Detection of *Salmonella:* It relies on detection of C8-esterase enzyme that is produced by *Salmonella* and can be detected by a chromogen 5-bromo-6-chloro-3-indolyl caprylate (magenta caprylate). Antibiotics such as cefsulodin and novobiocin are incorporated to inhibit growth of *Pseudomonas* and *Proteus.* Methylumbelliferyl-caprylate (MUCAP) is a test for detection of *Salmonella* based on detection of caprylate esterase. (Manafi, 1996).

3.1.2.2. Detection of *Listeria monocytogenes*: These media functions on the basis of the enzyme production and sugar fermentation and may also be supplemented with antimicrobial agents to increase the selectivity. (Gasanov, Hughes, & Hansbro, 2005). One of the enzymes widely being used for the detection of the *Listeria* is phosphatidylinositol-phospholipase C (PIPL-C). (Jamali, Ching, & Lin, 2013).

3.1.2.3. Detection of *Bacillus cereus*: There is a chromogenic medium called as Brilliance Bacillus cereus agar (formerly called as Chromogenic Bacillus cereus agar) which has a chromogenic substrate that results in formation of blue/green colonies. Scientists have also reported the use of a chromogenic substrate for the demonstration of sphingomyelinase activity in human brain. (Hydrolysis, 1983).

3.1.3: Disadvantages of cultural methods:

They need longer detection times nearly about five to seven days (Rajapaksha & Elbourne, 2018). However, these methods are still used because of their high selectivity and specificity (Law, Mutalib, Chan, & Lee, 2015)

3.2. Culture independent detection methods:

3.2.1. Molecular detection methods: These detection methods are highly specific as they can detect specific nucleic acid sequences in the target organism by hybridization with short synthetic oligonucleotide. These include polymerase chain reaction (PCR) and Loop Mediated Isothermal Amplification (LAMP). These detection methods are rapid and gives results within 4-24 hours. Nucleic-acid based methods have been used for detection of organisms *Vibrio cholerae, Bacillus cereus, Escherichia coli* and *Staphylococcus aureus Bacillus anthracis,*

Salmonella sps, Pseudomonas aeurginosa, Escherichia coli, Listeria monocytogenes (Law et al., 2015).

3.2.2.Immunoassays: Immunological methods for detection include ELISA, Oligonucleotide DNA microarray, Immunomagnetic Separation based methods Immunochromatographic strips. These methods have been used for the detection of pathogens like *Salmonella typhi, Listeria monocytogenes, Camplylobacter jejuni, Bacillus cereus, Shigella flexneri, Escherichia coli, Staphylococcus aureus, etc*

3.2.3: Disadvantages of culture independent methods:

The detection time of these tests can vary from minutes to hours, this gives an upper hand to these tests over traditional culture-based methods. The disadvantages of the process like it being expensive, dependency on skilled labour and infrastructure are inherent to the process. (Manual & Methods, 2009)

4. Portable culture devices:

These devices are used for point of care (POC) diagnosis and for detection of pathogens in resource limited settings. They employ the principle of molecular marker based or culture-based detection systems. These devices follow the ASSURED criteria, which means that they should be Affordable, Sensitive, Specific, User friendly, Rapid and robust, Equipment free and Delivered to the needy. Along with being inexpensive in mass production it is also useable by low skilled personnel on site. Maribel Funes-Huacca *et al* have demonstrated design of a culture device consisting of medium impregnated on paper sheets, a PDMS layer and tape which can be used to cultivate and enumerate *E.coli* in remote resource limited settings (Funes-huacca, 2012). A PCD for Antimicrobial susceptibility assays which function on similar lines as the Kirby–Bauer disc diffusion test has been reported to be used for *Escherichia coli* and *Salmonella typhi* (Funes-huacca, 2013). Lokur A. reported proof of concept studies, which demonstrates the use of PCD's for detection of various organisms like *Staphylococcus aureus, Escherichia coli* from various food samples. (Lokur A, 2018)

IV. AIM AND OBJECTIVES:

Aim: Standardization of portable culture device for detection of microbes.

Objectives:

Phase I: Optimization of portable culture device for detection of microbes

To optimize portable culture device for the detection of microorganisms like *Salmonella typhi, Listeria monocytogenes, Bacillus cereus, Escherichia coli, Staphylococcus aureus, etc*. with respect to

a) Type and concentration of chromogenic substrate
b) Media optimization
c) Specificity testing
d) Reproducibility
e) Sensitivity
f) Stability

Phase II: Proof of concept studies using standard cultures

Phase III: Proof of concept studies using food or water samples

To test the device for detection of microbes from spiked food and water samples

To test the device for detection of pathogens using actual food and water samples

Phase IV: Field testing of the fabricated device

To test the device for field application

V. Materials and methods:

1. **Fabrication of portable culture devices:**

   Portable culture device (PCD) will be developed using Whatman filter paper number 1, thin PDMS sheets and masking tape as described by Funes-Huacca (2012) with some modifications (A. Lokur, 2018)

2. **Optimization of detection of test organisms on Portable Culture Device:**

   The fabricated portable culture device will be tested for their ability to detect standard test organisms using chromogenic substrate. Standardization will be carried out with respect to the following points:

i. **Substrate:** Substrate specific for detection of test organisms will be tested with other organisms to prove the specificity. The organism specific substrate of standardized concentration will be used for further tests. Examples of substrates that can be used for detection include Chlorophenol red-β-D-galactopyranoside (CPRG) and 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-GlcA, X-Gluc) for coliforms and *Escherichia coli* respectively.

ii. **Time of Detection:** Saline suspensions having varying cell numbers of test organisms will be inoculated on medium impregnated PCD. Time required to obtain positive results will be standardized and matched with the cell number. The detection time and cell numbers will be correlated. Cell number in the dilutions will be validated by viable count. The optimized time will be used for further tests.

iii. **Specificity:** Specificity of the device will be computed using number of true positives and false negatives

iv. **Sensitivity:** Sensitivity will be determined as limit of detection (LOD). Dilutions of test organisms will be tested on PCDs using their chromogenic substrates. Cell number in the dilutions will be validated by viable count. The lowest dilution showing visible color distinguishable from blank will be reported as limit of detection.

v. **Reproducibility:** Experiments will be repeated multiple number of times so as to determine the reproducibility of the method.

3. **Study of device stability and shelf time:** It is important to determine the stability of the reagents and the device. Hence, the device will be stored for varying days at different temperatures. Post storage the devices will be used for detection of test organisms using the above standardized method.

4. **<u>Standardizing the device for detection of test organisms using spiked samples:</u>**

   Device will be used to detect the test organisms in spiked samples. The samples selected could be from food or water. The samples will be processed using a standardized protocol. The presence of the test organisms from the samples will be detected using the developed detection system.

5. **<u>Testing the device for detection of test organisms using samples:</u>**

   The PCD standardized above will be tested using actual samples. The samples selected could be from food or water. The tests will be carried out in the laboratory and on field. Results obtained will be compared.

VI. Expected results:

The project aims at detection of microorganisms on portable culture device using chromogenic substrate. The time required of color development on the device should be proportional to cell number. The device is expected to detect test organisms from the samples that need to be tested at Point-of-Care or Point-of-Control (POC). Such detection systems would be ideal for detecting organisms on the field, in locations which lack centralized testing facilities and skilled manpower. Thus, the device is expected to be suitable for on-site applications.

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